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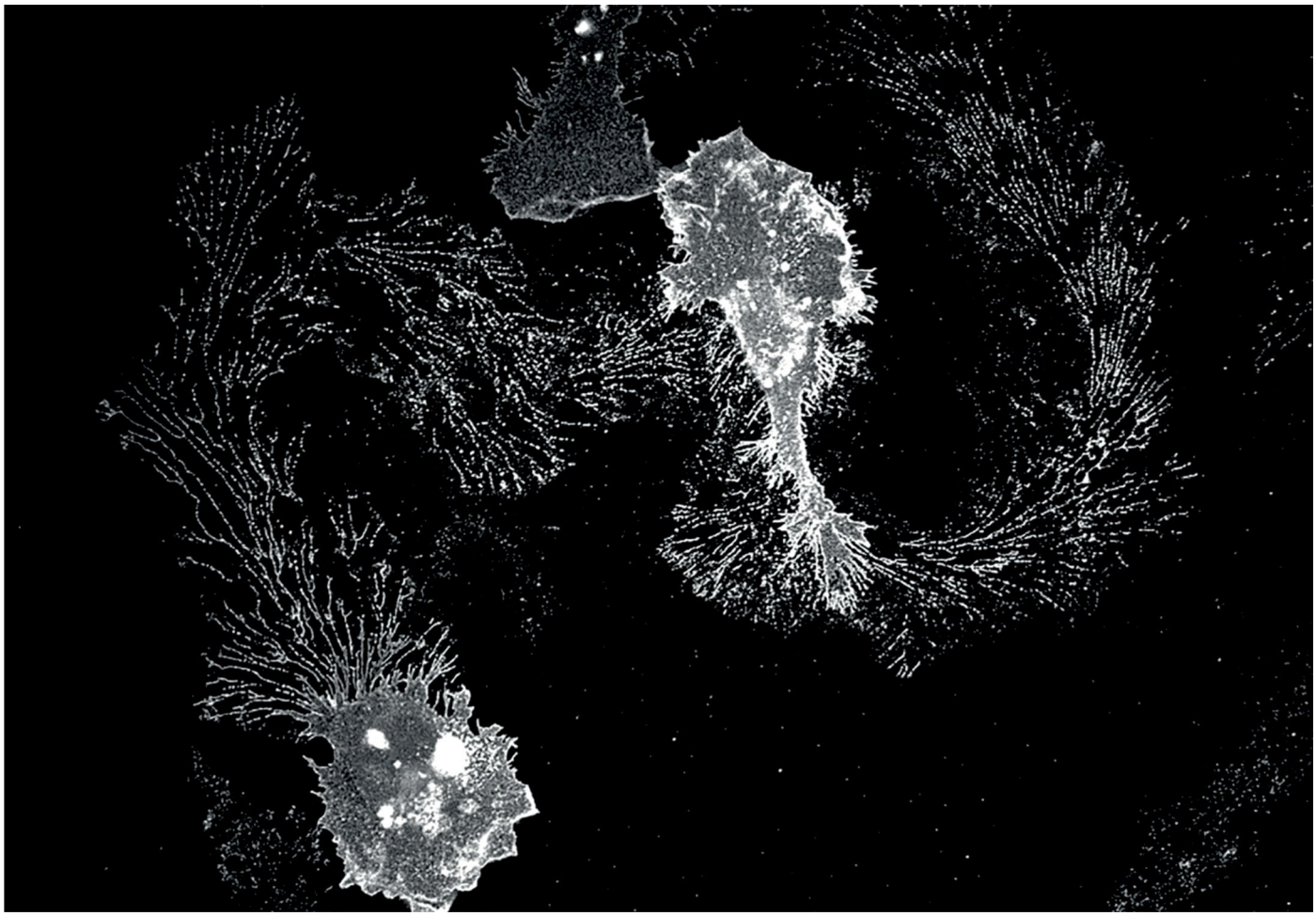

The pH-responsive fluorescent dye pHluorin_M153R-CD63 makes it possible to visualize EVs upon their release from cells.

Eavesdropping on extracellular vesicles

A rapidly evolving toolbox is helping researchers to get a handle on the biological and functional diversity of these ubiquitous – but still somewhat enigmatic – cell-secreted nanoparticles

By Michael Eisenstein

As recently as 20 years ago, researchers working on extracellular vesicles routinely struggled to convince their colleagues of the biological importance of these tiny membrane-bound bubbles. "We met so many skeptical people in everyday life, at meetings: 'Are they real? Are they just artifacts?'," says Edit Buzás of Semmelweis University in Budapest. This was despite the fact that scientists had been describing such vesicles since the 1960s, and assigning biological activity to them since the 1980s[1]. "A lot of people were saying that I was kind of a 'proctologist of the cell'," jokes Guillaume van Niel of INSERM in Paris, France. "But I didn't care .... I was one of the first to be able to see them by electron microscopy, so I could prove they do exist, and they certainly have a role."

Time has proven the skeptics wrong. Today, extracellular vesicles (EVs) are the focus of a vibrant field, with labs around the world striving to understand why and how cells release these tiny particles, as well as the physiological

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Juan Manuel Falcón's research group at the Centro de Investigación Cooperativa en Biociencias (CIC bioGUNE) in Derio, Spain.

impact of the proteins, nucleic acids and other biomolecules contained within. There is already considerable evidence that EVs can facilitate cell-to-cell communication in various settings, wherein vesicles discharged by one cell travel through the circulation – potentially over considerable distances – only to be taken up and 'decoded' by recipients elsewhere in the body.

This process may be relevant in diverse disease settings, including tumor growth and metastasis. Accordingly, many researchers see clear opportunities for detecting and monitoring cancer by intercepting these vesicular communiques. In 2020, for example, David Lyden at Weill Cornell Medicine in New York City and colleagues identified EV signatures that can provide effective early warning of developing tumors[2]. "Most of our patients were stage 1," says Lyden. "And we had very high sensitivities and specificities with the test – with greater than 90% accuracy, we can predict who has cancer or not." Other clinical research efforts are focused on turning naturally derived EVs into drug-delivery vehicles or therapeutics themselves.

This journey has not been entirely smooth sailing. Researchers have learned that the spectrum of EV subtypes is broader than initially recognized, and they are still grappling with how best to purify, analyze or even classify these diverse nano- and microparticles. Untangling their function has proven just as thorny, drawing on observations and inferences from a host of different in vitro and in vivo models with a variety of different imaging and analytical instruments. The good news is that the field is aggressively pushing for greater rigor and reproducibility, even as it arms itself with more powerful and reliable tools for untangling the enigmas of EV biology.

Blurred lines

The unifying feature of EVs as a category is that they are all lipid-membrane-enclosed particles, laden with protein, DNA, RNA or other biomolecules, that get released by cells into the extracellular environment. Early on, researchers settled on a fairly rudimentary classification schema. "You had the exosomes, which were small, and you had the microvesicles, which were really large," explains Kenneth Witwer, at the Johns Hopkins University School of Medicine. In many cases, the term 'exosome' was used as a catchall descriptor for EVs in general.

But it soon became clear that this was an overly simplistic and artificial divide. "It was just all about the techniques everybody was using," says Witwer. As of this writing, researchers have described roughly a dozen different types of EVs, and these categories and the means to selectively identify and classify them remain the subject of debate.

Even if size alone is not a defining characteristic, it can still offer useful signposts. For example, exosomes typically fall in the size range of 30–150 nanometers in diameter. But other, typically larger subsets of EVs can also yield particles falling within this size range, such as the 'apoptotic bodies' shed by dying cells, which can measure between 50 nm and 5 µm. As such, this offers only a rough starting point for categorization.

"I think the most scientific approach to this is in the biogenesis," says Buzás. From this perspective, EVs fall into two general buckets. Exosomes are those vesicles that originate from the cellular interior, as a product of the endosomal pathway that facilitates many important intracellular trafficking activities. In contrast, ectosomes are EVs that form via the bubbling out of the outer plasma membrane of the cell. These distinct routes of origin give rise to considerable differences in membrane composition and contents and are thus offer a useful dividing line, even if just as a starting point.

At present, exosomes remain by far the most well-characterized subset of EVs. "That's because that's where we've traced the biological activity to," says Vanderbilt University researcher Alissa Weaver. But most in the field suspect that there is a great deal still to be learned about the functional categories that might blur the lines between existing EV categories or lead to a more biologically grounded approach to classification. Van Niel draws the comparison to how immunologists looked at lymphocytes before immunohistochemistry and flow cytometry. "We are at the point now where you might imagine just sorting white blood cells by their size and density," he says.

In pursuit of purity

To get at the defining characteristics for a given pool of EVs, researchers need the means to selectively isolate particular subsets of these nanoparticles. Many methods are now available, but each entails trade-offs in terms of ease of use, throughput and reproducibility.

Witwer describes the options in terms of a 'specificity/recovery matrix', where high performance on one axis means sacrifices on the other. "You have some methods that are very good at recovering everything in the sample – and that might be fine," he says. One such method entails using reagents like polyethylene glycol to precipitate out the suspended nano- and microparticles from a given sample. But this also brings down non-vesicular

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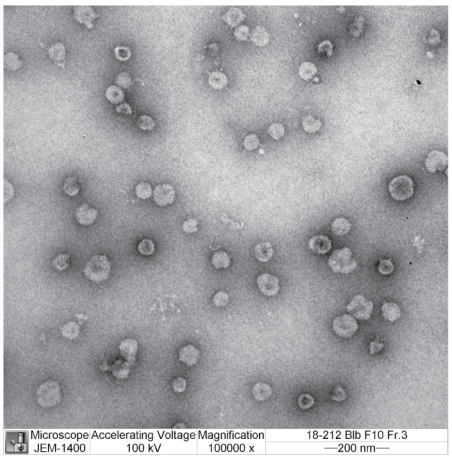
proteins, RNAs and other debris, and although this might not be an issue in the context of biomarker discovery or analysis, it is ill-suited for selective characterization of subsets of EVs.

The most common starting point for EV purification at present is differential ultracentrifugation, in which highly heterogeneous preparations of cell culture medium or other biological specimens are spun down at increasing speeds to selectively precipitate particles of a particular size. For exosomes, this is typically a multi-step process in which the EVs themselves are ultimately pelleted at forces upwards of 100,000 times the force of gravity for many hours. "This is very reproducible," says Juan Manuel Falcón, a researcher at CIC bioGUNE in Derio, Spain. "The problem is that it is too laborious."

And if the goal is to achieve truly pure preparations of a particular vesicle subtype, it is often only a first step in the process, as the products of differential ultracentrifugation inevitably remain contaminated with other biomolecules. This is particularly problematic with blood products like plasma and serum, which are rife with proteins that co-enrich with exosomes in differential ultracentrifugation — most notably low- and high-density lipoproteins, which are similar in size and density to many EV subpopulations. Buzás also notes that they are several orders of magnitude more abundant than vesicles in blood-derived samples. Complementary methods can help in terms of achieving superior purification, including density-gradient centrifugation and size-exclusion chromatography — strategies that allow more precise separation of EVs from other contaminant molecules.

Newer alternatives are emerging as well. For example, Lyden's group has been working with a technique called asymmetric flow field-flow fractionation (AF4), in which a liquid sample containing ultracentrifugation-purified EVs is passed through a channel while also being subjected to a perpendicular fluid flow[3]. This results in the highly efficient size-based separation of particles as they traverse the channel while also preserving a lot of the heterogeneity inherent to the sample. "It's very good as an analytical tool … but it's still a bit labor-intensive," says Haiying Zhang, a Weill Cornell researcher who collaborates routinely with Lyden. Another promising platform, developed by Luke Lee at Harvard and Fei Liu at Wenzhou Medical University in China, uses a sophisticated ultrafiltration method to purify EVs directly from clinical samples such as urine, saliva or plasma. In a 2021 publication, they showed that their EXODUS platform could extract EVs from a urine sample within ten minutes while achieving yield and purity superior to those of a range of other well-established isolation methods[4].



Electron micrograph of small exosomes purified from cultured cells via differential ultracentrifugation and AF4.

Finally, there are opportunities to selectively isolate vesicular subpopulations via affinity purification with antibodies that specifically recognize known EV-specific surface markers. The more popular targets here are three transmembrane proteins belonging to the tetraspanin family: CD63, CD81 and CD9. "That's an opportunity to definitively purify vesicles," says Buzás. But even here, there is considerable complexity. For example, Koen Breyne, a researcher in Xandra Breakefield's lab at Harvard Medical School, notes that although tetraspanins are a defining feature of EVs, not all EVs produce them at equivalent levels. As such, even affinity capture experiments will inevitably yield an incomplete picture of the vesicle population. "I think one statistic cannot identify a certain class or type of EVs," says Breyne.

A molecular manifest

Profiling the molecular contents of EVs is generally a more straightforward task. "You can do any kind of analysis, including proteomics, lipidomics and RNA sequencing," says Weaver. "It's just a matter of getting enough vesicles and getting it pure enough."

Mass spectrometry (MS) is currently the tool of choice for deciphering the protein, lipid, carbohydrate and metabolite composition of an EV preparation with high sensitivity and broad coverage. But achieving reproducibility with such MS analyses remains a challenge, according to Lyden. "If we sent the same sample to two different places, there would be maybe 700 proteins detected at one and 1,000 detected at another," he says, although his group is now exploring new molecular labeling strategies that could help to overcome this problem.

There is also the issue of discriminating biomolecules that are truly associated with vesicles from impurities in the sample that get nonspecifically extracted alongside the EV prep. "For example, there's a lot of extravesicular RNA ribonucleoprotein complexes in serum," says Weaver, "and you do have to worry about contamination of the outside of a vesicle." If the specific aim of the analysis is to get at the molecules residing at the core of a vesicle, this may not be a problem. "You can distinguish internal cargo from surface-associated molecules by, for example, digesting with nucleases or glycosidases or proteases," says Buzás. But surface molecules also cannot be ignored, as they can inform as to the EV's ability to interact with and initiate a biological response in target cells.

This situation is further complicated by the recent realization that EVs may be naturally surrounded by a biomolecular 'corona' of proteins and other molecules. "Previously, a lot of components were considered awful contaminants of EV preparations," says Buzás. "But now we come to the understanding that, oops, they are actually external cargo or some natural components of the vesicles." These are not covalently linked to or embedded in the vesicle membrane, but instead form a more weakly attached outer coating, and this means that overzealous efforts to generate a pure preparation may end up unwittingly stripping away essential information. "The methodologies that we use for exosomes are very strong, and keeping the corona intact is difficult." says Falcón. As such, although a growing number of researchers now believe that corona analysis will be an important aspect of understanding EV function, the field is still struggling with how best to manage this fragile outer shell.

Respecting individuality

Bulk EV preparations will offer at best a gestalt view of the vesicular population present in a sample. Hakho Lee of Massachusetts General Hospital points out that a milliliter of human plasma can carry on the order of 10 billion EVs, and MS analysis of these might reveal thousands of different proteins and other biomolecules. "But looking at the individual particles, clearly there are subpopulations," says Lee. "Analyzing those subpopulations might tell us about the genesis or the origin of those EVs

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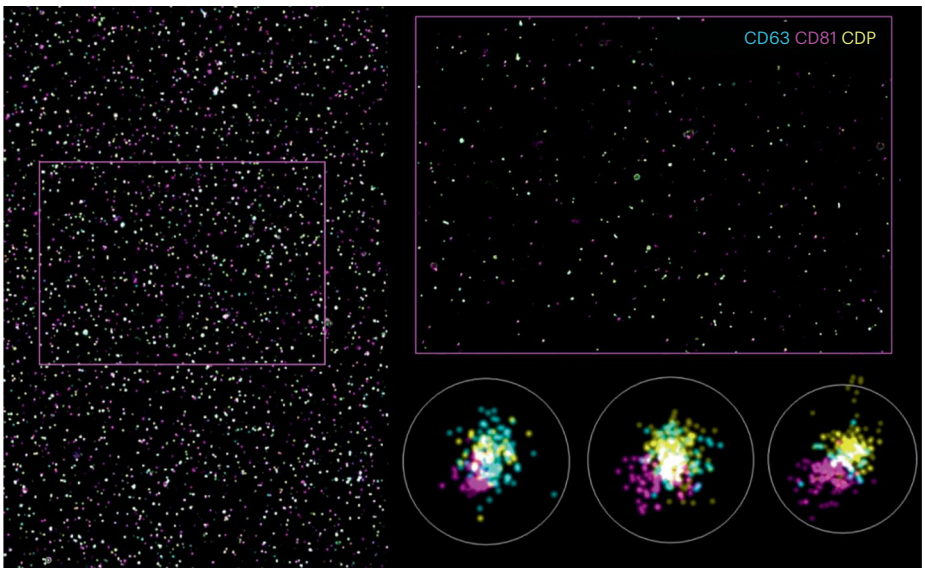

Super-resolution microscopy imaging of purified fibroblast EVs (left and top right) with the ONI Nanoimager, with individual tetraspanin distribution at the single-EV level (bottom right).

from different cell types." Accordingly, there has been considerable interest in technologies that provide detailed information about single vesicles.

Falcón's team was among the first to use cryo-electron microscopy for such analyses, and it is now among the more well-established methods for inspecting individual vesicles. Here, EV-containing samples are rapidly frozen under conditions that trap the biomolecular structure intact, after which the sample can be imaged via transmission electron microscopy without the need for further treatment or labeling. "You see good information on morphology, size and also something about the contents," says Falcón. "You can observe vesicles that are well loaded because they are electron-dense." However, this approach is low-throughput and cannot provide specific information about what biomolecules are associated with a given vesicle.

A method called Raman tweezers microspectometry can provide more insight in this regard. In this method, a tightly focused laser is used to both trap vesicles for analysis and also collect Raman spectral data that reveal the lipid, nucleic acid or protein composition of a given EV. "It's limited because it doesn't tell you what the protein is, but at least it allows you to do some kind of classification," says Falcón.

Other methods make more detailed molecular profiling possible — so long as researchers know what markers they want to look for. There is considerable excitement around flow cytometry for the marker-based analysis of EV populations, for example. A 2019 study from a multinational team led by Bernd Giebel and André Görgens at University Hospital Essen in Germany demonstrated the feasibility of using imaging flow cytometry to sort individual EVs on the basis of a combination of a fluorescently labeled tetraspanin marker and labeled antibodies that recognize other EV surface proteins[5]. Commercial kits are now available that enable more extensively multiplexed flow cytometric analyses of EV subsets. The MACSPlex kit developed by Miltenyi Biotec can track up to 37 different surface markers. Weaver sees this as an exciting tool for single-EV analysis, but adds that the method is still in the development phase. "The challenge for flow cytometry is sensitivity," she says.

Super-resolution imaging is even allowing researchers to peer inside the vesicle. Lyden is among a handful of researchers who have begun using ONI's benchtop Nanoimager instrument to study individual EVs using stochastic optical reconstruction microscopy (STORM). In STORM, small subsets of individual fluorophores are excited in individual snapshots and then either switched back to a dark state or photobleached. By repeating this process for several rounds, one can reconstruct fluorescence images with resolution below the diffraction limit of light. "You can actually examine how well is your protein represented in a hundred vesicles … [and] is it found only on cancer exosomes and not on, say, immune cell exosomes?" says Lyden. STORM even makes it possible to visualize distributions of proteins and whether a molecule of interest is dispersed or clustered — potentially indicating activation.

But even single-vesicle detail doesn't mean easy answers. Witwer notes that the small number of molecules in a single EV can make it hard to get insights into the function of a particular cargo of interest. "You would have to look at maybe a thousand EVs before you find a specific microRNA — or ten thousand," he says. And for now, such throughput is out of reach of single-vesicle analysis platforms.

Caught in the act

Frozen snapshots of isolated vesicles will only tell you so much about real-world biology. Fortunately, researchers are making considerable headway in developing tools for exploring the biogenesis, release and uptake of EVs in living cells and tissues.

This has necessitated steady evolution of the labeling toolbox. Until relatively recently, imaging experiments relied primarily on lipid-based dyes that generically label membranes. These can be useful, particularly for shorter-term experiments, but many of these dyes have the potential to transit from one membrane to another and can even form independent aggregates that linger in the system, creating deceptive artifacts. "You're not following an EV — you are following a dye," cautions van Niel, adding there is the risk that such dyes might alter the chemical properties of the EV lipid membrane to an extent that affects vesicle function.

Genetically encoded labels can confer greater control over the extent and specificity of labeling. If the goal is still to visualize the membranes themselves, one can engineer cells to express fluorescent proteins that incorporate sites for palmitoylation, such as the PalmtdTomato reporter developed by Breakefield and Charles Lai of the Academia Sinica in Taiwan[6]. This post-translational fatty acid modification facilitates insertion into lipid membranes and labels these surfaces with greater fidelity than standard lipid dyes. Fluorescently labeled tetraspanins are another popular tool for robust and selective EV labeling. "They are not easy to manipulate, but I know for sure that what I'm working with is made of membrane," says van Niel. However, there is also the risk that overexpression of these modified tetraspanins might alter EV behavior.

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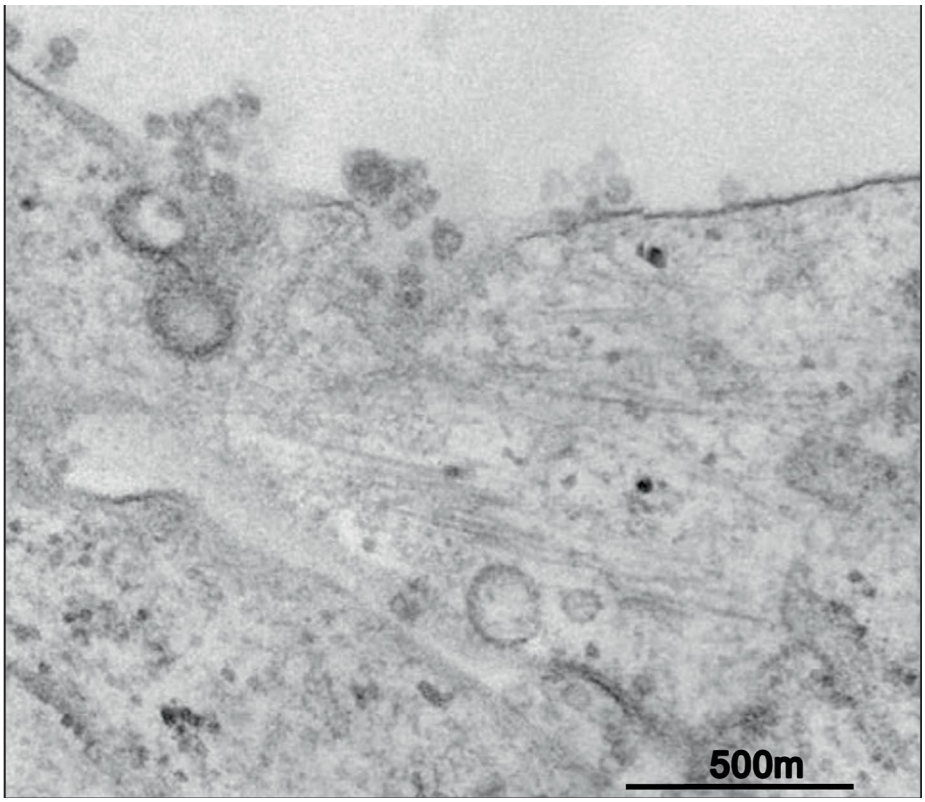

Electron microscopy captures an exosome secretion event.

Other labeling strategies operate in an even more selective fashion. For example, Weaver's team has developed genetically expressed labels in which the tetraspanin CD63 is coupled to the pH-responsive pHluorin reporter, which remains dark as long as the labeled EVs are within the acidic environment of the cellular endosomal machinery[7]. "This shows you the moment when the exosomes leave the acidic compartment, and once they are released from the cells by exocytosis, all of a sudden they start to glow," says Buzás. "That's spectacular."

Keeping it real

Cell culture is a great way to access EV biology with high spatial and temporal resolution in a tightly controlled fashion. "You can learn a lot about how different cells take up the vesicles and what the fate is of the cargo," says Breakefield. But one must be cautious in attempting to generalize these results to in vivo reality. For example, Falcón has found that cultured cells or tissues often exist in a more stressed state, in which they are overly active in terms of secretion and uptake of EVs. "You can have an idea of the mechanism that could be behind EV biogenesis," he says. "But my view is that still they are not physiological – it is still the cells responding to a situation that is not normal."

A number of groups are performing in vivo studies in rodents – for example, infusing batches of labeled vesicles and then determining their final destination in the body, or using genetically encoded markers to selectively label EVs originating from certain cell types. This can be a challenging task with fluorescent markers, however, which can only be imaged in tissues that are relatively close to the skin and accessible to intravital microscopy or by directly analyzing the tissue after surgical removal. "Most people – and we do it most of the time too – just take the organs out," says Breyne.

Bioluminescence-based luciferase reporters offer an alternative that makes it possible to image the movement of EVs deep within the body using instruments such as the PerkinElmer IVIS instrument. But this approach still suffers from poor resolution due to the small size of individual vesicles, which results in a weak signal. "For a cell to light up … it means that hundreds of these EVs have gone into that cell," says Lyden. This can be useful for characterizing defined vesicle preparations, but is unlikely to offer much clarity on routine cell-to-cell signaling in vivo.

Van Niel is among a growing number of researchers who have turned to zebrafish as an appealing alternative. This species is evolutionarily closer to humans than other microscopy-friendly models like *Drosophila* or *Caenorhabditis elegans*, with the added virtue that its larvae are see-through. This makes it possible to do in vivo fluorescence imaging experiments with single-vesicle resolution. A pair of 2019 studies led by van Niel, his INSERM colleague Jacky Goetz, and Frederik Verweij, now at the University of Utrecht, demonstrated the power of this approach[8,9]. "We could see that the biogenesis mechanisms in vivo in zebrafish were the same as the ones observed in vitro in murine and human cells," says van Niel. "We were the first to be able to clearly see single EVs behaving in the blood flow, and finally, where they go in vivo."

Even so, there is sufficient evolutionary distance from humans – including the absence of organs like the lungs or prostate gland – for researchers to be cautious about overgeneralizing these findings to humans. But Witwer is excited about this model's potential to provide previously inaccessible insights into the real-world physiological behavior of EVs, and he sees important complementary progress in methods for studying EV dynamics in nonhuman primates with radiological imaging that could ultimately aid in translating experimental EV insights into the clinic. "This year alone, there were at least three papers that have come out about biodistribution of EVs in nonhuman primates," he says.

Building confidence

The surge of interest in EVs has generated a tidal wave of publications: the annual output of PubMed records mentioning exosomes or EVs jumped from just 342 in 2011 to nearly 8,000 in 2021. But this frenzy of research has also created concerns about the level of experimental quality and reproducibility in this nascent field. "There's a lot of literature out there that I certainly don't believe," says Breakefield. "The problem is that the field is so hot … that just encourages people to do very quick experiments and to interpret them however they want."

In 2014, the International Society for Extracellular Vesicles embarked on an effort to introduce a higher degree of quality control to the field. The result was a set of guidelines on how researchers should go about providing 'minimal information for studies of extracellular vesicles' (MISEV) from their studies, along

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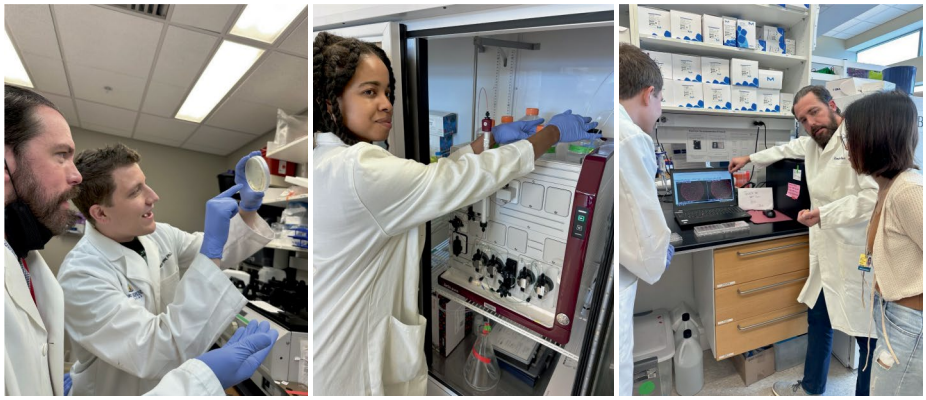

The Witwer lab at Johns Hopkins University School of Medicine uses a variety of technologies to study extracellular vesicles.

with recommendations to enable greater consistency and reproducibility in EV preparation, characterization and testing. The initial MISEV document was updated in 2018 with extensive feedback from hundreds of researchers[10], and a new iteration is in the works.

"I think it was a game changer," says Buzás, who has been part of MISEV since the beginning. "Every newcomer who steps in the field can just follow those basic guidelines and has a much easier way to go." MISEV's recommendations are not meant to be ironclad rules, and Breakefield points out that it would be extremely challenging — if not impossible — for the research community to completely standardize its methods given the diversity of experimental procedures, technologies and sample types now in use. But adherence to the guidelines can give readers more confidence in a study's robustness. An Hendrix of Ghent University in Belgium and colleagues developed a database called EV-TRACK that can help EV researchers to share their methods and assess their adherence to transparency and reproducibility standards, including those proposed by MISEV. As of this writing, experimental details from more than 2,300 publications have been submitted to the database.

While basic and clinical researchers continue to converge on best practices, efforts to commercialize EV-based technologies are barreling forward. And some researchers are concerned that for many of these companies, their grasp may be exceeding their reach — particularly with EV-derived therapeutics. "I just think they're not that close to a product by a long shot," says Breakefield. But other areas of commercial development, such as EV-based diagnostic biomarkers, are making more headway, and overall, Witwer is enthusiastic about the possibility of real clinical progress in the near future. "There was a lot of skepticism in the past, and that's healthy," he says. "But now there's more money flowing in, there's more resources to develop these things, and I think it's happening."

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